Short communication

Determination of the activity of ponazuril against *Sarcocystis neurona* in cell cultures

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Abstract

The present study examined the efficacy of ponazuril in inhibiting merozoite production of *Sarcocystis neurona* in cell cultures. Ponazuril inhibited merozoite production by more that 90% in cultures of *S. neurona* treated with 1.0 μg/ml ponazuril and greater than 95% inhibition of merozoite production was observed when infected cultures were treated with 5.0 μg/ml ponazuril. Ponazuril may have promise as a therapeutic agent in the treatment of *S. neurona* induced equine protozoal myeloencephalitis (EPM) in horses. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses caused primarily by infection with *Sarcocystis neurona*. The condition has been recognized for over 20 years (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974) and EPM is the most important protozoal disease of horses (MacKay et al., 1992) . Recent serological surveys using the Western blot test indicate that 45–53% of horses in North America have antibodies to *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Salville et al., 1997).

Exposure of horses in Argentina and Brazil to *S. neurona* is estimated at 36% using the same Western blot test (Dubey et al., 1999a,b). Sporocysts excreted by opossums (*Didelphis virginiana*) are the source of *S. neurona* infection (Dubey and Lindsay, 1998).

Treatment of EPM is often with pyrimethamine combined with trimethoprim and sulfonamides (MacKay et al., 1992; Lindsay and Dubey, 1999). Additional effective agents are needed to treat EPM. In the present study, we examined the activity of ponazuril against *S. neurona* in cell cultures to determine if it might be effective in vivo.

2. Materials and methods

2.1. S. neurona isolates and cell culture

S. neurona merozoites (SN6 strain), isolated from a horse with EPM (Dubey et al., 1999c), were grown and maintained in bovine turbinate (BT cells, ATTC CRL 1390, American Type Culture Collection, Rockville, MD, USA) or African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, MD, USA) as described previously (Lindsay et al., 1999). The host cells were grown to confluence in $25\,cm^2$ plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell cultures were maintained in growth medium in which the FBS content was lowered from 10 to 2%. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

For quantitative studies, merozoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and magnesium. The host cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10 ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 μm filter to remove cellular debris. The number of merozoites in the filtrate was determined using a hemacytometer. The final volume of suspension was adjusted so that 2.5×10^5 merozoites were present for inoculation.

For general maintenance of merozoites, monolayers were examined with an inverted microscope for the development of lesions (areas devoid of host cells caused by parasite replication) in the monolayer or the presence of many extracellular merozoites. Once lesions were observed or many extracellular parasites were present, the monolayer was scraped with the tip of a 5 ml pipette and 1–3 drops of the merozoite containing fluid was transferred to two flasks of BT cells. Merozoites of *S. neurona* were passaged in this manner every 3–7 days.

2.2. Cell culture assay

The activity of ponazuril was determined in a merozoite production (MP) assay (Lindsay and Dubey, 2000). Ponazuril was dissolved in DMSO to make a stock solution of 1 mg/ml. Dilutions were made from this stock solution and the highest concentration of DMSO in any solution was 0.01% (v/v). Cell monolayers were inoculated with 2.5×10^5 merozoites of *S. neurona*. Two hours after inoculation, the medium was removed and replaced with

Table 1
Percent reduction in merozoite production in cell cultures infected with *S. neurona* and treated with various concentrations of ponazuril

Concentration of		% Reduction
Ponazuril (μg/ml)	Merozoite count±S.D.[a]	
CV-1 cells		
None	1,406,438±276,817	NA
0.1	412,062±102,227	70.7
1.0	139,125±200,878	90.1
5.0	40,125±43,758	97.1
BT cells		
None	4,175,125±464,794	NA
0.01	3,040,688±410,257	27.2
0.1	231,813±93988	94.4
1.0	63,313±46,594	98.5
5.0	86,375±58,853	97.9

[a] S.D.: standard deviation.

maintenance medium containing ponazuril at various concentrations (μg/ml) (Table 1). Control flasks received maintenance medium with 0.01% DMSO only. Four flasks were used per ponazuril treatment dose. The merozoite production assay was conducted after 10 days of treatment. This exposure time was chosen so that three asexual generations of the parasite (Lindsay et al., 1999) would be exposed to the ponazuril treatment. At 10 days of treatment the amount of monolayer destruction was visually estimated in the living cultures using the phase optics of the inverted microscope. The medium was removed from the cultures, the volume was recorded, and the number of merozoites determined by counting in a hemacytometer. The total number of merozoites present in each flask was determined by multiplying the volume of medium by the numbers of merozoites (mean of 16 counts per treatment[4 counts per flask])/ml of medium. The percentage reduction in merozoites due to treatment was calculated by subtracting the mean treated values from the mean control value, dividing this numerator by the mean control value and multiplying the product by 100. Experiment 1 was done in CV-1 cells and Experiment 2 was done in BT cells.

The following procedure was used to determine if ponazuril treatment killed developing stages of *S. neurona*. After the medium was collected for the MP assay, the cell monolayer was rinsed twice with maintenance medium to wash off any residual ponazuril and 5 ml of maintenance medium was added to the flask. The flasks were then examined for 30 days for renewed growth of parasites, or monolayer destruction, or both.

3. Results

In Experiment 1, the CV-1 cells in flasks infected with *S. neurona* and not treated with ponazuril had approximately 30–50% monolayer destruction when the MP assay was conducted; whereas those treated with 0.1, 1.0 or 5.0 μg/ml ponazuril had 20–30, 10–30 and 5–10% monolayer destruction, respectively. In Experiment 2, the BT cells in flasks infected

with *S. neurona* and not treated with ponazuril had approximately 30–50% monolayer destruction when the MP assay was conducted; whereas those treated 0.01, 0.1, 1.0 or 5.0 μg/ml ponazuril had 30–40, 5–10, and <5% monolayer destruction, respectively. Ponazuril had good activity at 0.1–5 μg/ml (Table 1). Ponazuril inhibited development but did not completely inhibit merozoite production at the levels tested in both CV-1 and BT cell cultures. Renewed multiplication occurred in *S. neurona* treated flasks when the ponazuril containing medium was removed.

4. Discussion

Ponazuril is a major metabolite of the triazine anticoccidial toltrazuril. Toltrazuril is effective against intestinal coccidiosis in avian (Chapman, 1987, 1989) and mammalian species (Gjerde and Helle, 1991; Driesen et al., 1995). It is also effective against hepatic coccidiosis in rabbits (Peeters and Geeroms, 1986). Ponazuril most likely acts on the apicoplast of *S. neurona* because the triazine anticoccidials appear to act on this organelle (Hackstein et al., 1995). The apicoplast is a vestigial, nonphotosynthetic plastid described in apicomplexan parasites (Köhler et al., 1997). This plastid-like organelle is not found in vertebrate cells making it an excellent drug target (Fichera and Roos, 1997; Roberts et al., 1998).

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